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High load hepatitis B virus replication inhibits hepatocellular carcinoma cell metastasis through regulation of epithelial–mesenchymal transition

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SUMMARY

Objectives: The aims of this study were to investigate the effect of hepatitis B virus (HBV) replication on the metastatic ability of hepatocellular carcinoma (HCC) cells and to explore a potential mechanism from the perspective of epithelial–mesenchymal transition (EMT).
Methods: Two short-interfering RNAs (siRNAs) against the HBV S gene were used to inhibit HBV replication in HepG2.2.15 cells. To evaluate the level of HBV replication and interference efficiency, HBV antigen and HBV DNA were detected by ELISA and quantitative PCR (Q-PCR). Invasion and metastatic abilities were compared between different groups by wound healing and trans-well assays. Immunofluorescent staining and Western blotting were utilized to detect EMT markers.
Results: Both siRNAs effectively inhibited HBV replication in HepG2.2.15 cells. Compared to control HepG2.2.15 cells, cells transfected with the siRNAs showed characteristics of the mesenchymal phenotype and augmented their ability to invade and metastasize. Inhibition of HBV replication suppressed E-cadherin and induced a switch to vimentin expression. Western blots confirmed the decrease in E-cadherin expression. The level of E-cadherin expression was also lower in HepG2 cells than in HepG2.2.15 cells.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy and third leading cause of cancer-related death in the world.[1] A high potential for vascular invasion and metastasis of HCC is associated with a poor prognosis.[2] Hepatitis B virus (HBV) infection is the most important risk factor for HCC and is present in approximately 80% of HCC patients.

It has been reported that metastasis and recurrence is more common in HCC patients with an HBV infection than in those without.[3,4] HBV DNA integration, especially the HBV X gene, is regarded to be critical for these processes.[5,6] However, our previous studies have found that HepG2.2.15 cells that are transfected with HBV whole genome and support stable HBV replication, show decreased proliferation and metastatic ability compared to parental HepG2 cells.[7,8] Qiu et al. reported that HBV infection decreases the risk of liver metastasis in patients with colorectal cancer and elevates the surgical resection rate of liver metastases.[9] Zhou et al. found that the outcome of intrahepatic cholangiocarcinoma (ICC) patients with HBV infection is better than the outcome of patients without HBV infection after curative resection,[10] though HBV infection is an independent risk factor for

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ICC.[11] Thus, the real effect of HBV on migration and its related mechanisms is contradictory and remains unclear.

It is possible that the effect that HBV elicits in cells depends on its state. A high load of HBV replication may inhibit the metastatic ability of the host cell, while integration of only a part of the HBV gene into the host cell may lead to increased metastatic ability. In this study, a short-interfering RNA (siRNA) approach was used to inhibit HBV replication in HepG2.2.15 cells, and the metastatic ability of these cells was examined in comparison to HepG2.2.15 cells. Furthermore, the potential mechanism was explored from the perspective of epithelial–mesenchymal transition (EMT).

2. Methods

2.1. Cell line and cell culture

The HepG2.2.15 cell line supports stable HBV replication and protein expression, as well as the production of virus particles.[12] The cells were cultured in Dulbecco's modified Eagle medium (DMEM; Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 100 μg/ml streptomycin, 100 IU/ml penicillin, and 380 μg/ml G418 (Invitrogen) at 37 °C in a 5% CO_2 incubator.

2.2. siRNA and transfection

Two siRNAs were designed against the HBV S gene (GenBank accession number **U95551.1**); they were synthesized and purified by Invitrogen Co. (Shanghai, China). Their sequences are as follows: siRNA1 sense-GCGGGUAUAU UAUAUAAGATT, antisense-UCUUAUA UAAUAUACCCGCTT; siRNA2 sense-GGGACUUC AAUCCCAACAATT, antisense-UUGUUGGGAUUGAAGUCCCTT. The negative control (NC) was a scrambled sequence without any significance.

Transfection was performed using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instructions. HepG2.2.15 cells were seeded in 24-well plates with 5×10^4 cells per well, 1 day before transfection. The final concentration of siRNA was 20 nmol/l.

2.3. ELISA and Q-PCR

The HBV e antigen (HBeAg) in culture medium, which can reflect the level of HBV replication, was examined by ELISA (Intec, Xiamen, China). Meanwhile, the HBV DNA level in supernatant was assayed by fluorescence quantitative PCR (Q-PCR; PG Biotech, Shenzhen, China). These processes were performed in accordance with the manufacturer's instructions. The results were used to determine the level of HBV replication and the interference efficiency of the siRNAs in HepG2.2.15 cells.

2.4. Wound healing assay

After 24 h of transfection, the cells were grown to confluence and wounds were introduced using a pipette tip. The plates were washed twice with DMEM to remove detached cells and incubated in complete growth medium without FBS. The width was measured and photographs were taken immediately (0 h) and at 72 h after wounding. The recovery ability was represented as a percentage of the initial width.

2.5. Trans-well assay

After 24 h of transfection, the cells were trypsinized for the trans-well assay. In brief, complete medium (600 μl) was added to the lower compartment of an invasion chamber, and 5×10^4 cells in 200 μl serum-free medium were added to the upper chamber. After 72 h of incubation, the migrated cells were fixed and stained with hematoxylin and eosin, and the number was counted in five randomly selected power fields under light microscopy.

2.6. Immunofluorescent staining

After 72 h of transfection, the cells seeded on a coverslip were fixed with 10% formalin and permeabilized with 0.1% Triton X-100. E-cadherin (CST, MA, USA) and vimentin (ZSGB-BIO, Beijing, China) antibodies were incubated with the cells at 37 °C for 1 h. Fluorescein-labeled anti-rabbit and Texas red-labeled anti-mouse secondary antibodies (Vector, Burlingame, CA, USA) were used against the respective primary antibodies. 4′,6-Diamidino-2-phenylindole (DAPI) was used to stain the nucleus.

2.7. Western blot analysis

After 72 h of transfection, the protein extracted from cells was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with E-cadherin antibody at a dilution of 1:800 overnight at 4 °C and subsequently incubated with alkaline phosphatase (AP)-conjugated secondary antibodies (ZSGB-BIO) for 1 h at room temperature. Immunoreactive bands were detected using Western Blue (Promega, Madison, WI, USA). β-Actin (ZSGB-BIO) was used as the internal control.

2.8. Statistical analysis

All data were presented as the mean ± standard deviation. The two groups were compared with the Student's *t*-test. A *p*-value of <0.05 was considered statistically significant.

3. Results

3.1. Both siRNAs can inhibit HBV replication in HepG2.2.15 cells

As shown in Figure 1a, HBeAg was stably produced in HepG2.2.15 and NC cells, while it was significantly decreased in the cells transfected with siRNA1 and siRNA2, by ELISA. The HBV DNA was also distinctly reduced in siRNAs-treated cells compared to HepG2.2.15 and NC cells by Q-PCR (Figure 1b). The two siRNAs against the HBV S gene effectively inhibited HBV replication in HepG2.2.15 cells, and there was no significant difference between their interference efficiency.

3.2. Decreased HBV replication induced cellular morphological changes

Morphological features were characterized by inverted microphotography (Figure 2). HepG2.2.15 cells were oval and lacked processes. They grew in multiple adherent layers and exhibited epithelial phenotypes. However, HepG2.2.15 cells transfected with siRNAs were relatively scattered and had extended processes, which are characteristic of a mesenchymal phenotype. This suggested that the cells transfected with siRNAs were relatively invasive.

3.3. Inhibition of HBV replication augmented the metastatic ability of HepG2.2.15 cells

The results of the wound-healing assay demonstrated an augmented migration capacity in HepG2.2.15 cells transfected with siRNAs when compared to the blank control and the NC group

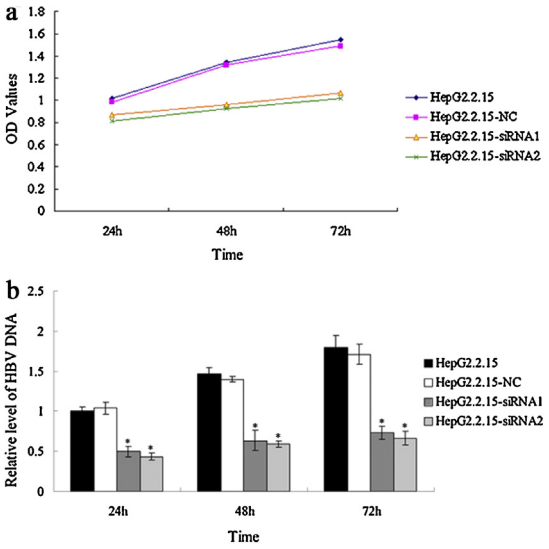

Figure 1. Determination of the interference efficiency of the siRNAs. (a) Detection of HBeAg by ELISA. The level of HBeAg in supernatant was significantly decreased in HepG2.2.15 cells transfected with siRNA1 and 2 compared to blank (HepG2.2.15) and negative control (HepG2.2.15-NC) cells. (b) Detection of HBV DNA levels by Q-PCR. The measured HBV DNA levels were normalized to the level of HepG2.2.15 cells at 24 h. The HBV DNA in supernatant was distinctly reduced in siRNA-treated cells compared to HepG2.2.15 and NC cells. Compared to HepG2.2.15 cells, $^{*}p < 0.01$.

(Figure 3a, b). Meanwhile, the siRNA2 group was more invasive than the siRNA1 group ($p < 0.05$), although both siRNAs presented a similar interference efficiency in HepG2.2.15 cells. More cells transfected with siRNAs migrated through the pores of the membrane and entered the lower side of the invasion chamber than in the control group (Figure 3c, d). Trans-well analysis demonstrated that both siRNAs enhanced the invasive ability of HepG2.2.15 cells. These results suggested that invasion and metastasis was increased when HBV replication was inhibited by HBV siRNAs in HepG2.2.15 cells.

3.4. E-cadherin was decreased after HBV replication inhibition by siRNAs

According to the results of the immunofluorescent staining, the epithelial marker E-cadherin was highly expressed in HepG2.2.15 and NC cells, whereas the level of the mesenchymal marker vimentin was low. The expression of both markers was reversed when HBV replication was inhibited in HepG2.2.15 cells by either siRNA (Figure 4). Meanwhile, the results of the Western blot assays also confirmed the higher level of E-cadherin expression in HepG2.2.15 and NC cells compared to those transfected with siRNA1 or 2 (Figure 5). Additionally, HepG2, which is the parental cell line of HepG2.2.15 and has a relatively higher invasive potential, also showed lower expression of E-cadherin.

4. Discussion

Although significant research has focused on the role of HBV in HCC, the exact function and mechanism by which HBV functions remains unclear. Most studies have focused on the effect of part HBV gene integration into DNA of the host cell, whereas only a few have investigated HBV whole genome integration or HBV replication. This study primarily explored the relationship between HBV replication and the invasive potential of the host cells, based on our previous findings.

It has been indicated that RNA interference (RNAi) can inhibit HBV replication effectively in cell culture and in vivo and is believed to have therapeutic potential for HBV infection.[13,14] A number of studies have verified that specific siRNAs targeting four open reading frames (ORFs) of HBV are efficient at inhibiting the expression of viral antigens and the replication of HBV DNA.[15–18] Fu et al. designed and evaluated 23 siRNAs targeted throughout all four ORFs of HBV. The results showed that most of them were effective, but the siRNAs targeting different ORFs could lead to different efficacy in inhibiting HBV expression and replication.[19] It has been found that the joint use of siRNAs can provide a more powerful tool for the treatment of viral infection.[20] The therapeutic potential of siRNAs should be explored further. In this study, two siRNAs were designed and synthesized against the HBV S gene to inhibit HBV replication in HepG2.2.15 cells. It provided a good model to study the effect of HBV load on HCC cells.

To study the effect of HBV replication on the migration of HCC cells, we compared the invasive and metastatic potential of HepG2.2.15 cells to cells transfected with HBV siRNAs. The results indicated that HCC cells showed increased migratory ability when HBV replication was inhibited by siRNA. Increasing evidence suggests that EMT plays a critical role in the dissemination of cancer cells during HCC progression,[21,22] implicating a potential mechanism. EMT is a reversible process by which epithelial cells undergo a phenotypic conversion and assume a mesenchymal cellular phenotype, including elongated morphology, enhanced migratory and invasive ability, decreased expression of epithelial markers (such as E-cadherin and β-catenin), and increased expression of mesenchymal components (such as vimentin and fibronectin).[23,24] Many factors can contribute to the invasion and metastasis of HCC by inducing EMT processes, including hypoxia, microRNAs, and some protein molecules.[25–27] The tumor micro-environment in HCC has been identified as one of the most potent inducers of EMT and its reverse process mesenchymal–epithelial transition (MET).[28] In HBV-associated HCC, FAT10 has been found to induce EMT and promote invasion of cancer cells.[29] Suppression of E-cadherin is at the center of the EMT process.[30,31] E-cadherin is the major mediator of cell adhesion in adherent junctions. Translation factors, including Snail, Twist, and Zeb1, can regulate the expression of E-cadherin by binding the E-box sequence in the promoter region of CDH1.[24]

In this study, inhibition of HBV replication induced the suppression of E-cadherin and turned on the expression of

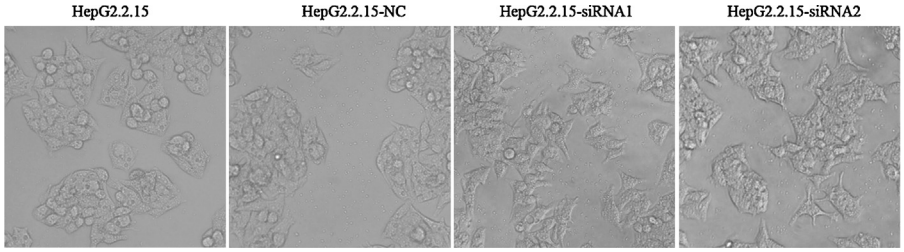

Figure 2. Morphological features of the different cellular subgroups. HepG2.2.15 cells transfected with siRNAs showed a relatively mesenchymal phenotype compared to blank control and negative control (NC) cells, which demonstrated a more epithelial phenotype.

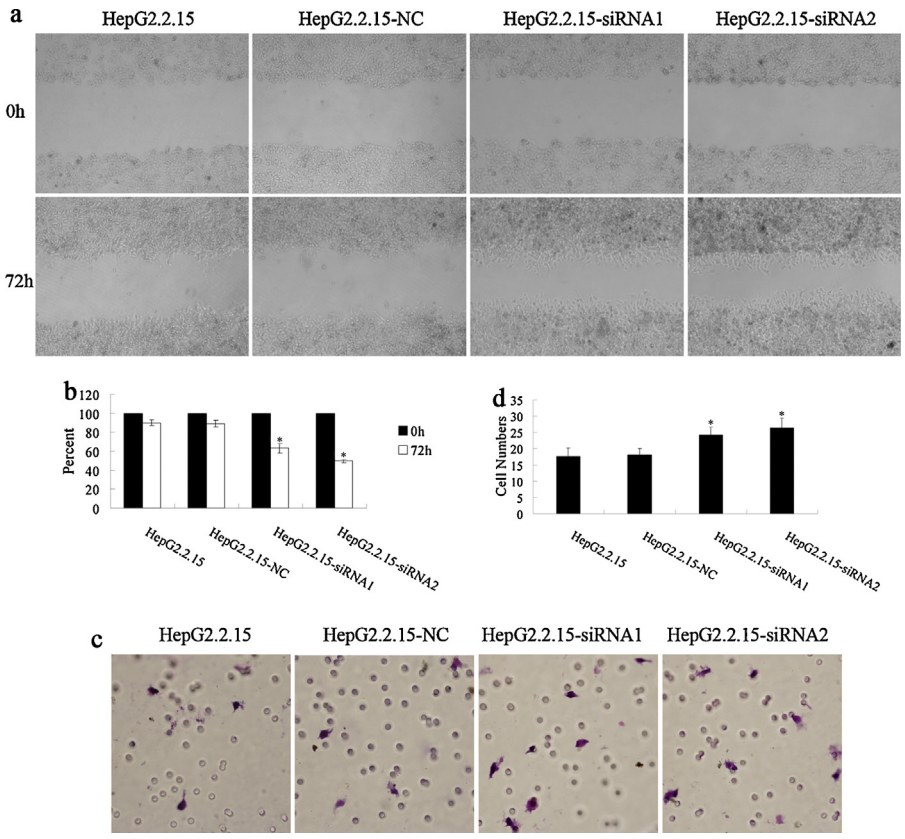

Figure 3. Assay of invasive and metastatic ability. The cells in the siRNA1 and 2 groups demonstrated an augmented migration capacity when compared to blank control and negative control (NC) cells. (a) Wound-healing assay. (b) The recovery ability of the cells to the wound-healing assay is represented as a percentage of the initial width. (c) Trans-well assay. (d) The average number of migratory cells under a high power field is demonstrated by the bar graph. Compared to HepG2.2.15 cells, $^{*}p < 0.01$.

vimentin. The result of the Western blot assays confirmed the decreased expression of E-cadherin in HepG2.2.15 cells transfected with siRNAs. Our previous study found that the parental cell line HepG2 is more invasive than HepG2.2.15.[8] This study verified a lower level of E-cadherin expression in HepG2 cells. Thus, HBV replication might affect the migratory ability of host cells by inducing the reverse process of EMT, especially the augmentation of E-cadherin expression. Additionally, HepG2.2.15 cells transfected

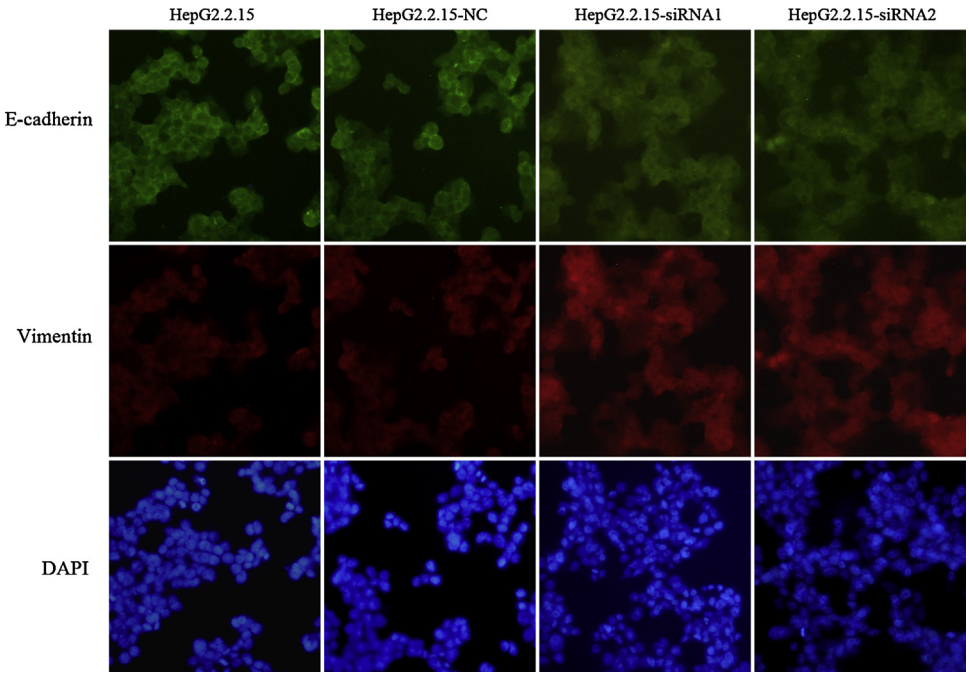

Figure 4. Immunofluorescent staining. Inhibition of HBV replication suppressed the epithelial marker-E-cadherin and turned on the expression of the mesenchymal marker vimentin.

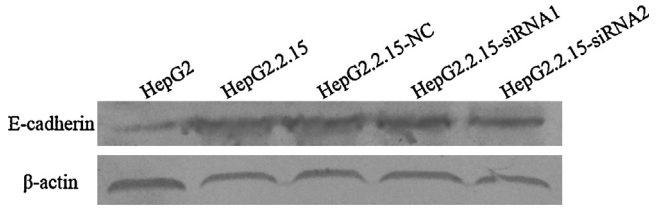

Figure 5. Western blot assay. The level of E-cadherin in HepG2.2.15 and negative control (NC) cells was higher compared to cells transfected with siRNA1 or 2 ($p < 0.05$). E-cadherin was significantly increased in HepG2.2.15 cells compared to HepG2 cells ($p < 0.01$).

with siRNA showed relatively scattered and more frequent processes, which is consistent with a mesenchymal phenotype, providing additional evidence for the presence of EMT.

In conclusion, the siRNA approach was effective at inhibiting HBV replication. A high load of HBV replication inhibited the invasive and metastatic ability of host cells by regulating EMT, primarily by upregulating the expression of E-cadherin. Integration of the HBV whole genome might produce a different effect on the migratory ability of host cells compared to the integration of a part of the HBV gene. Additionally, this result may explain to some extent why only a small percentage of patients with HBV infection will progress to HBV-related HCC.

Acknowledgements

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Conflict of interest: No conflict of interest to declare.

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